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Cloning and Characterization of Partial cDNAs for Woodchuck Cytokines and CD3ϵ With Applications for the Detection of RNA Expression in Tissues by RT-PCR Assay

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Immunologic reagents and methodology are essential to develop further the woodchuck and woodchuck hepatitis virus (WHV) as a model of immune response, inflammation, and immunotherapy in hepatitis B virus (HBV) infection. Partial cDNA clones for the woodchuck CD3ϵ marker of T cells (536 bp) and for selected woodchuck cytokines were developed, including IL-1β (332 bp), IL-2 (249 bp), IL-4 (205 bp), IL-10 (476 bp), IFN-γ (476 bp), and TNF-α (381 bp). This panel of markers includes sets to measure RNAs for T cells (CD3ϵ), immune response induction (IL-1β, IL-2), TH subsets (TH1, IL-2/IFN-γ vs. TH2, IL-4/IL-10), and effector molecules that regulate hepadnavirus replication and liver injury (IFN-γ, TNF-α). Primers representing highly conserved segments of genes from other species were used to derive the partial cDNA clones. Target RNA was obtained from woodchuck peripheral blood mononuclear cells (PBMC) that were stimulated in vitro with ConA, LPS, and human rIL-2. The cDNA clones were validated by 1) comparison with other species for homologies in the nucleotide and predicted amino acid sequences and 2) a first generation assay demonstrating induction of the respective RT-PCR products in stimulated woodchuck PBMC. The corresponding RNAs were also detectable in most cases in the total RNA from the livers of uninfected and WHV-infected woodchucks and differential expression of IFN-γ and TNF-α RNAs was suggested. Second generation, semi-quantitative assays for the RNAs were validated using RT-PCR and dot-blot hybridization with ^{32}P-oligomers derived from the internal sequences of the respective clones. Continued study of the woodchuck immune response to WHV infection using these assays will provide insight into the kinetics and immune mechanisms that initiate and maintain chronic hepadnavirus infection and, hence, enable development of improved immunotherapies for established chronic HBV infection. ***J. Med. Virol. 53:85–95, 1997.***

KEY WORDS: woodchuck; WHV; cytokine; T cell marker; RT-PCR assay

INTRODUCTION

The eastern woodchuck and the woodchuck hepatitis virus (WHV) represent a potentially useful model for the study of immunologic responses to hepatitis B virus (HBV) infection, and their role in outcome and disease processes [Cote and Gerin, 1996]. Elucidation of immune mechanisms that initiate and promote chronic WHV infection can facilitate development of rational immunotherapeutic approaches to eradicate established chronic HBV infection and interdict disease sequelae, including hepatocellular carcinoma (HCC). Assays for cellular immunologic responses in woodchucks, such as lymphocyte proliferation and IL-2 production, have been used to study lymphocyte responses to mitogens, cytokines, superantigen, alloantigen, and viral antigens [Cote and Gerin, 1995; Menne et al., 1997], including epitope-specific responses of T helper cells [Menne et al., 1997]. The cDNAs for the

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woodchuck IL-12 heterodimer have been cloned and sequenced [Ackrill et al., Gene Bank Nos. X97108, X97109], which will facilitate modeling with this cytokine in woodchucks. Compared to humans and mouse models, however, assays for woodchuck cytokines, lymphocyte markers, cytotoxic T lymphocyte (CTL), and major histocompatibility complex (MHC) are underdeveloped. Such methodology is essential to advance further the woodchuck as model of immune response, inflammation, and immunotherapy in human HBV infection.

In this study, we extended the range of assay formats for woodchuck immune responses. Partial cDNA clones and RT-PCR-based assays were developed for the RNAs of selected woodchuck cytokines and a lymphocyte marker (e.g., CD3ϵ). The specificity and sensitivity of RT-PCR-based assays were validated for detecting immunocytokine RNA expression in blood and liver compartments during WHV infection. This approach permits a direct analysis of RNA transcripts and RNA-related events at relevant sites of WHV infection and immune response, as modeled in other systems [Fukuda et al., 1995; Kim et al., 1995; Larrea et al., 1996; Shindo et al., 1996; Sieling et al., 1994; Svetic et al., 1991, 1993; Yamamura et al., 1992]. The present sequence information will also facilitate the development of woodchuck-specific cytokines and antibodies for use for assay development, mechanistic studies, and therapeutic modeling.

MATERIALS AND METHODS

Isolation and Standardization of RNA From Woodchuck PBMC

Woodchuck peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-hypaque purification from whole blood (EDTA-treated). PBMC from WHV-negative woodchucks were cultured in flasks at 10^6/ml as described by Cote and Gerin [1995] and stimulated for 2 to 3 days with concanvalin A (ConA) (20 μg/ml), lipopolysaccharide (LPS) (40 μg/ml), and human rIL-2 (100 IU/ml). Stimulated PBMC were centrifuged (1,000 rpm, 7 min, room temperature), the supernatants were harvested, and the cell pellets were processed further to obtain RNA (RNeasy Total RNA Kit; Qiagen, Chatsworth, CA). Briefly, cell pellets were lysed and homogenized with 0.6 ml of lysis buffer provided with the kit. The lysates were applied onto the RNeasy spin column and washed under the appropriate conditions. Bound RNA was eluted in 30 μl of sterile RNase-free water, quantified by optical density (OD_{260}), and divided into smaller aliquots (stored −130°C). Total RNA isolated by this method was of sufficient quantity and quality (range, OD_{260}/OD_{280} = 1.7–2.2) for use for isolating cDNA clones and as a standard for RT-PCR assays.

Primer Pairs and RT-PCR Amplification

Primer pairs used to derive the primary clones of IL-1β and IFN-γ were human sequences purchased from Continental Laboratory Products, Inc. (San Diego, CA). Primer pairs used to derive the clones of the other woodchuck cytokines and CD3ϵ were synthesized based on conserved sequence regions matched among human, porcine, bovine, ovine, feline, canine, and murine species; the specific primer sets were as follows: IL-2 (human sequences), IL-4 (human), IL-10 (mouse, rat), TNF-α (mouse), and CD3ϵ (sheep). CD3ϵ is hereafter referred to CD3. Primer pairs corresponding to woodchuck-specific sequences were synthesized subsequently, where indicated, after the primary clones were sequenced.

Primer pairs were screened by RT-PCR using a hot-start tube format. The input RNA was standardized RNA from stimulated normal woodchuck PBMC. Samples of total RNA (0.01–1 μg/μl) were reverse-transcribed (15 min, 37°C; 15 min, 42°C) using 1 μl (100 U) of moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The RT buffer mix consisted of 50 mM Tris-HCL (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphates, 0.6 U/μl ribonuclease inhibitor, and 0.4 μg/μl of random hexamers (Boehringer Mannheim, Mannheim, Germany) (10 μl total volume/reaction). Tubes were cooled and the wax layer was overlaid with 40 μl of PCR buffer mix containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM deoxynucleoside triphosphates, 0.4 μM of each primer, and 0.05 U/μl of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The PCR reactions were amplified in a Perkin-Elmer thermocycler (35 cycles; 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min).

In each experiment, reactions were controlled by adding enzymes, primers, and buffer mix to a water sample containing no RNA, and also by RT-PCR of the sample RNA using β-actin primers that were conserved among several species: forward 5′-primer, 5′-TGGA-ATCCTGTGGCATCCATGAAAC; reverse 3′-primer, 5′-TAAAACGCAGCTCAGTAACAGTCCG (349 bp product).

Cloning and Characterization of Partial cDNAs for Cytokines and CD3

PCR products of the expected size for IL-1β, IL-2, IL-4, IL-10, IFN-γ, and CD3 were cloned into the PCR II vector using the TA cloning kit (Invitrogen, San Diego, CA). The TNF-α PCR product was cloned into pAMP1 using the Clone Amp kit (GIBCO BRL), following appropriate modification of the primers according to the kit instructions. RT-PCR products were isolated from agarose gels, ligated to the respective vectors, and used to transform *E. coli*, DH5α (PCR II), or INVαF′ (pAMP1), which were then selected by ampicillin resistance and lacZ expression (X-gal). The clones were sequenced using an automated DNA sequencer (Applied Biosystems) by Sanger's fluorescent dideoxy-chain termination method. One clone of IL-2, one clone of IFN-γ, and two or more clones of the other markers were isolated and sequenced from pooled cDNA that was made in five to 10 individual RT-PCR reactions involving each primer set. Only two discrepancies were found among IL-10 clones among the total number of nucleo-

tides sequenced, and this was resolved in favor of the most common representative. The sequences were validated for open reading frames, and the nucleotide and predicted amino acid sequences were further compared for homologies with gene bank sequences available from other species.

RT-PCR Detection of Cytokine and CD3 RNA Induction in Woodchuck PBMC

Induction of cytokine and CD3 RNAs was demonstrated in cultured woodchuck PBMC. RNAs were isolated as described above using the RNeasy kit (Qiagen) and quantified by OD_{260}. Six WHV-negative woodchucks weighing >4 kg were sampled for 40 ml of whole blood (EDTA-treated). PBMC were isolated by Ficoll-hypaque centrifugation and adjusted to 10^6 cells/ml in complete medium. Day 0 (D0) RNA was isolated from 5 × 10^6 PBMC from each animal (yield, 450–900 ng RNA/10^6 PBMC); day 0 represented 18–24 hr from the time of blood sampling and overnight shipment from the woodchuck facility. The remaining PBMC were divided equally into six treatment groups per animal for sampling of stimulated and unstimulated cells at three subsequent time points (two animals/stimulator). Paired unstimulated cultures were processed in parallel as controls (yield, 400–750 ng RNA/10^6 PBMC seeded). Culture supernatants and total PBMC RNA were obtained on days 1, 2, and 3 following stimulation with ConA (20 μg/ml) or LPS (40 μg/ml), and on days 1, 3, and 6 following stimulation with human rIL-2 (100 IU/ml; Cetus, Inc.). At the end of the stimulation period, yields of RNA from ConA-, LPS-, and IL-2-stimulated PBMC, respectively, were 4,700, 450, and 2,700 ng total RNA per 10^6 PBMC seeded. Under the above stimulation conditions, these amounts of RNA reflect mainly an increase of RNA per cell rather than an increase in cell number [Cote and Gerin, 1995]. RNA samples for the respective treatment groups were assayed by RT-PCR at constant concentrations. PCR products were analyzed on 2% agarose gels by ethidium bromide (ETBR) staining.

RT-PCR Detection of Cytokine and CD3 RNA in Woodchuck Liver

Liver tissue was obtained surgically from uninfected and WHV infected woodchucks during the mid-acute phase (week 14) following neonatal inoculation with WHV7P1 [Cote and Gerin, 1996]. Snap frozen liver tissue (150–1,000 mg wet weight) was homogenized mechanically in 1 to 4 ml of 4 M guanidine-isothiocyanate containing 0.1 M 2-β-mercaptoethanol, 25 mM citrate, and 0.5% sarcosine. The sample was sheared and total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [Chomczynski and Sacchi, 1987]. Care was taken to remove only the upper two-thirds of the aqueous phase to prevent contamination of the RNA by DNA at the interface. Total RNA was precipitated from the aqueous phase following addition of an equal volume of cold isopropanol (4°C, 15,000 rpm, 30 min). The final RNA pellet was washed with 80% ethanol, air dried, resuspended in 200–700 μl of sterile distilled RNase-free water, quantified by optical density (OD_{260}), and divided into smaller aliquots (40–150 μl, stored −130°C). These amounts of RNA correspond to a relatively large fraction of liver tissue. The total liver RNA obtained was of sufficient quantity and quality for detection of various immunocytokine RNAs by RT-PCR when assaying 0.3–3 μg of liver RNA and analyzing the products by ETBR staining on agarose gel.

Semi-Quantitative RT-PCR Assays of Cytokine and CD3 RNA

A semi-quantitative RT-PCR assay was evaluated first by assay of woodchuck IL-2 RNA synthesized by in vitro transcription of the cloned fragment. Briefly, a linear template of the clone was prepared by HindIII digestion and RNA was transcribed by T7 polymerase (Promega, Madison, WI) (37°C, 2 hr). The sample was then digested with DNase and the RNA was extracted and its quantity determined by OD_{260}. This standard was diluted in distilled water in serial 10-fold increments to well beyond the calculated endpoint. One microliter of each dilution was then amplified by RT-PCR using appropriate primer pairs. Fifteen to 20 μl of the resulting standard product was analyzed on agarose gel by ETBR staining, and the remaining portion was used for dot blot hybridizations.

For dot blot hybridizations, one-tenth volume (5 μl) of the final RT-PCR reaction mixture was denatured in 100 μl of 1 M NaOH, blotted onto nylon membrane filters (0.2 μ, Schleicher and Schuell, Keene, NH), and cross-linked by UV irradiation. The filters were prehybridized (6 × SSC, 1% SDS, 5 × Denhardt's solution), and then hybridized at 52°C with a 5′-end-labeled ^{32}P-oligomer diluted in 6 × SSC/1% SDS. The oligomer consisted of an internal portion of the selected woodchuck cytokine or T cell marker. The oligomer was labeled with ^{32}P using T4 polynucleotide kinase [Sambrook et al., 1989]. After hybridization, filters were washed at 52°C with 2 × SSC/0.1% SDS, visualized on autoradiographic film, and counted using an AMBIS radiographic imager.

The semi-quantitative assay format was evaluated further using standardized PBMC RNA preparations. Briefly, RNA from stimulated PBMC (20–50 ng/μl) was diluted serially in distilled water in threefold increments to well beyond the estimated endpoint for cytokine RNA detection. One microliter of each dilution was amplified by RT-PCR using appropriate primers for IL-2 or IFN-γ. Products were then analyzed as described above using the respective 5′-end-labeled ^{32}P-oligomers.

RESULTS

Nucleotide Sequence Analysis of Partial cDNA Clones for Woodchuck Cytokines and CD3

Nucleotide sequences of the partial cDNA clones and respective primer sets are given in Figure 1. The IL-10

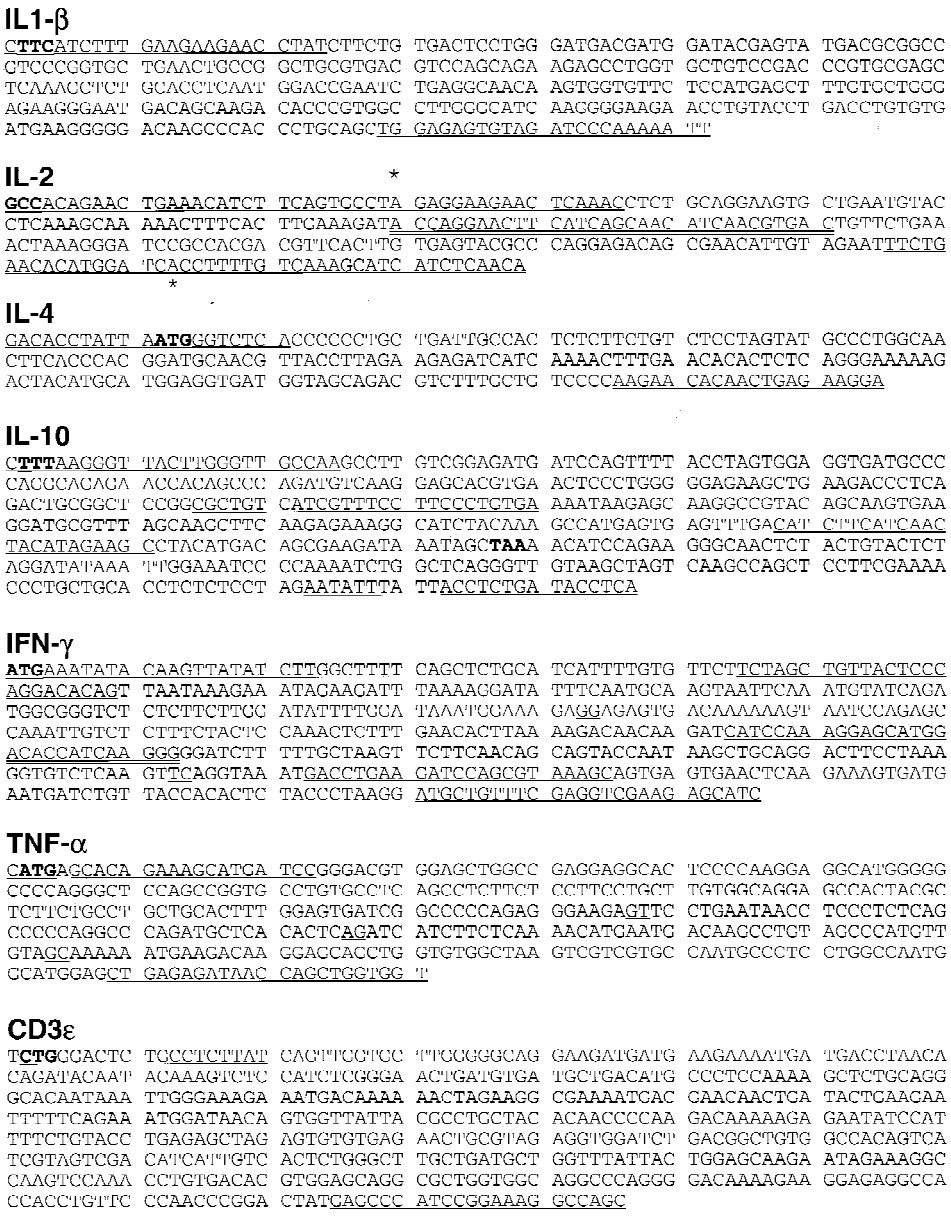

Fig. 1. Sequences of partial cDNA clones for woodchuck cytokines and CD3ε. First generation primers at the 5′ and 3′ ends of sequence are underlined. Bold trinucleotide is the first in frame codon for protein sequence or stop codon, where applicable. Double underlined sequences were used to develop ^{32}P-labeled oligomers. First internal primer for IL-10 (underlined) represents 5′ primer for 3′ overlapping clone; second underlined internal primer for IL-10 represents 3′ primer for 5′ overlapping clone; both are corrected to the woodchuck sequence. Internal 5′ and 3′ primers for IFN-γ (underlined) and IL-2 (underlined, starting at *) were used for second generation woodchuck-specific RT-PCR assays of the RNAs. Underlined dinucleotide sets in IFN-γ and TNF-α represent probable splice junctions for exons 1–4, respectively.

sequence is a composite derived from two overlapping clones and the internal primers shown are corrected to woodchuck-specific sequences. Nucleotide sequences of the woodchuck cytokines and CD3 had interspecies homologies ranging between 64% and 94%. Analogous regions of the sequences were evident with high homology to enable matching identifications with the corresponding gene bank sequences (not shown). The woodchuck-specific internal primers synthesized for IL-2 and IFN-γ were used to enhance the sensitivities of the respective RT-PCR assays. The probable splice junctions in IFN-γ and TNF-α are shown.

Amino Acid Sequence Analysis and Relative Positions of Partial Clones in the Protein Sequence

Predicted amino acid sequences for the partial cDNA clones are given in Figure 2. For three of the clones (IL-4, IFN-γ, and TNF-α), there was sufficient interspecies conservation near and within the signal se-

IL-1β

FIFEEEPIFC DSWDDDGYEY DAAVPVLNCR LRDVQQKSLV
LSDPCELKAI HLNGPNLRQQ VVFSMSFVLG EGNDSKTPVA
LGIKGKNLYL TCVMKGDKPT LQLESVDPKN

IL-2

ATELKHLQCL EEELKPLQEV LNVPQSKNFH LKDTRNFISN
INVTVLKLKG SATTFTCEYA QETANIVEFL NTWITFCQSI
IST

IL-4

MGLTPLLIAT LFCLLVCPGN FHGCNVTLEE IILTLNTLSG
KKTTCMEVMV ADVFAVPKNT TEK

IL-10

FKGYLGCQAL SEMIQFYLVE VMPQAENHSP DVKEHVNSLG
EKLKTLRLRL RRCHRFLPCE NKSKAVQQVK DAFSKLQEKG
IYKAMSEFDI FINYIEAYMT AKINS--

IFN-γ *

MKYTSYILAF QLCIILCSSS CYSQDTVNKE IEDLKGYFNA
SNSNVSDGGS LFLDILDKWK EESDKKVIQS QIVSFYSKLF
EHLKDNKIIQ RSMDTIKGDL FAKFFNSSTN KLQDFLKVSQ
VQVNDLKIQR KAVSELKKVM NDLLPHSTLR MLFRGRRA

TNF-α

MSTESMIRDV ELAEEALPKE AWGPQGSSRC LCLSLFSFLL
VAGATTLFCL LHFGVIGPQR EEFLNNLPLS PQAQMLTLRS
SSQNMNNKPV AHVVAKNEDK EQLVWLSRRA NALLANGMEL
RDNQLVV

CD3ε

LGLCLLSVGA WGQEDDEEND DLTQIQYKVS ISGTTVMLTC
PPKALQGTIN WERNDKKLEG ENDEQLILKN FSEMDNSGYY
ACYTTPRQKE NTHFLYLRAR VCENCVEVDL TAVATVIVVD
IIVTLGLLML VYYWSKNRKA KSKPVTRGAG AGGRPRGQKK
ERPPPVPNPD YEPIRKGQ

Fig. 2. Predicted amino acid sequences of woodchuck cytokines and CD3ε. First generation primer-related sequences are underlined. –, C-terminus; *, probable site of native N-terminus in mature secreted form of protein. Underlined dipeptides in INF-γ and TNF-α represent residues at the respective splice junctions. For TNF-α, the probable domain structure from the N-terminal Met involves a combination signal sequence, transmembrane domain, and cleavage site (which releases the soluble active C-terminal fragment) that extends to the TL site (i.e., just before the LR junction). Double underlined internal polypeptide sequence in CD3 corresponds to the extracellular domain (preceded by signal sequence); the underlined dipeptide in CD3 corresponds to the junction between transmembrane and intracellular (C-terminal) domains. Note that the intron-exon structure of CD3 can vary considerably among species and is not depicted in this context.

quences to permit cloning of the respective N-termini. In general, the amino acid residues corresponding to all of the initial primer sets were highly conserved among other species, but these may or may not correspond to woodchuck-specific residues. The probable splice junctions and/or domain structures are shown for IFN-γ, TNF-α, and CD3. In the case of TNF-α, the cloned fragment corresponds mainly to the membrane bound segment of the 26 KD precursor from which the 17 KD soluble form is derived [Perez et al., 1990].

The relative proportions and locations of the partial clones with respect to the human proteins are given in

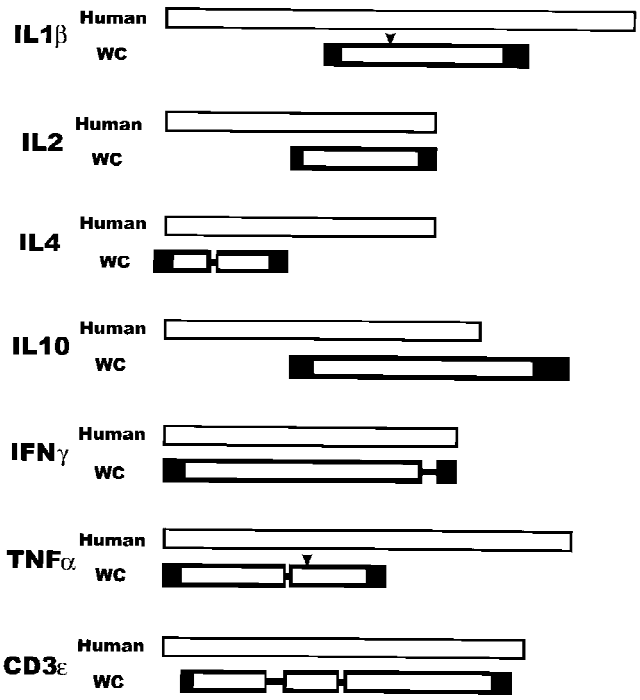

Fig. 3. Locations of partial cDNA clones for woodchuck cytokines and CD3ε in relation to coding sequences for the human proteins. Representations of the coding sequences for full-length human proteins are shown by bars with thin black line. Representations of the partial woodchuck clones are shown with a bold black line. The primer sequences are indicated by black bars on the woodchuck clones. A bold black line between two bars indicates a deletion (1 to 7 amino acids) in the woodchuck clone compared to the human; a vertical arrowhead indicates an insertion (1 to 2 amino acids) in the woodchuck clone.

Figure 3; Figure 3 also shows various insertions and deletions. The partial clones correspond to between 41% (IL-4) and 95% (IFN-γ) of the size of the respective human protein. The range of homologies for woodchuck sequences with those of human, mouse, and other species was 35–89%, with IL-10 showing the highest homology. Our near full-length clones for IFN-γ and CD3 had relatively low homology scores as compared with sequences from human and mouse (Fig. 4). Even though overall amino acid homologies were lower in these and other cases, note here that analogous regions of each sequence were evident with high homology to enable matching with the protein sequence bank.

Induction of Woodchuck Cytokines and CD3ε in Stimulated PBMC

In the following experiment we 1) demonstrate the specificity of primer sets by RNA induction and 2) validate a first generation RT-PCR assay for each cytokine RNA. Sizes of RT-PCR products corresponded to the size of the inserts described in Figure 1, except where indicated for IFN-γ (341 bp) and IL-2 (197 bp), which are the product sizes when using the woodchuck-specific primer sequences (Fig. 1). In the case of IL-10, the 303 bp 3′-end product was amplified using the woodchuck-specific 5′ primer (Fig. 1).

A composite figure for representative induction ex-

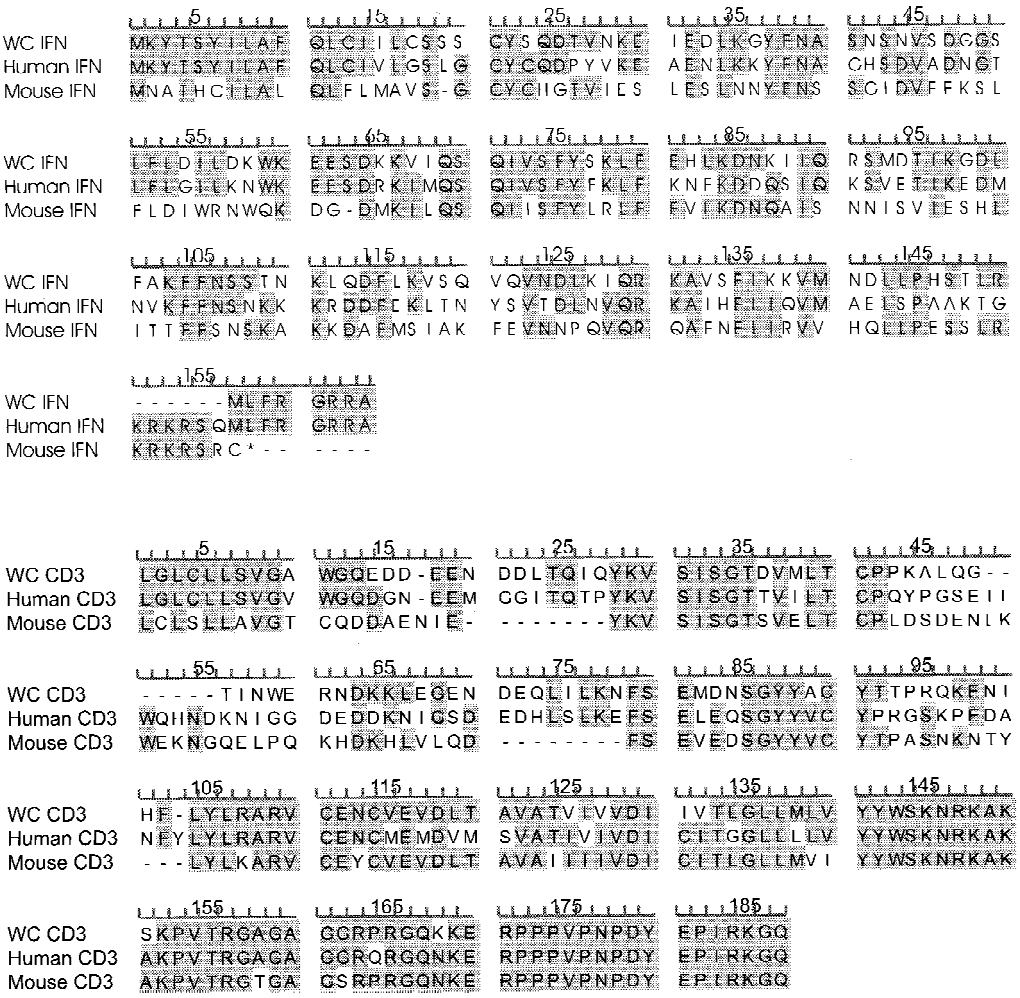

Fig. 4. Amino acid sequence homologies for woodchuck, human, and mouse IFN-γ and CD3ε. The alignment of woodchuck specific amino acid sequence (with regions corresponding to the primers) of these proteins is shown compared to those of human and mouse. Identical amino acid residues of human and mouse to those of woodchuck are indicated by shadowing. Deleted amino acid residues are shown by hyphens. *, C-terminus.

periments (Fig. 5) shows that stimulation resulted in specific increases in the respective target RNAs at nearly all time points when compared to unstimulated cultures and time zero RNA. Note here that total RNA input was adjusted up or down accordingly, depending on the primer set (range, 1–100 ng), in order to show differential detection of cytokine RNA relative to a constant signal for β-actin. Additional results are summarized in Table I using the same criteria for plus or minus induction depicted in Figure 5. ConA, a T-cell mitogen, specifically induced IL-2, IL-4, IL-10, IFN-γ, and TNF-α RNAs; induction of these cytokine RNAs occurred in parallel with induction of CD3 RNA (Table I, Fig. 5A) and detection of IL-2 in the culture supernatants (not shown; e.g., [Cote and Gerin, 1995]).

TNF-α and CD3 RNAs were highly detectable in as little as 50 ng of total RNA from unstimulated cells (data not shown), thus suggesting constitutive expression at a moderate level. The amounts of total RNA used in the above experiment (Table I, Fig. 5A) were reduced to 1 and 10 ng/reaction, accordingly, to show differential induction; but these two RNA species were still marginally detectable in unstimulated cells (Fig. 5A). IL-10 and IL-4 RNAs were also detectable in unstimulated PBMC at RNA inputs between 50 and 100 ng, respectively (not shown). Detection of IL-2 and IFN-γ RNAs in unstimulated PBMC using woodchuck-specific primers required up to 100–300 ng of RNA, thus suggesting low-level constitutive expression of these RNAs in unstimulated PBMC.

LPS, a rodent B cell mitogen and macrophage activator, specifically induced IL-1β RNA at days 1 and 2 of culture; this cytokine was detected at a lower level in 10 ng of time zero RNA (Fig. 5A). LPS did not specifically induce IL-2 production, IL-2 RNA, or CD3 RNA compared to unstimulated controls, consistent with its inability to stimulate T cells (Table I, Fig. 5A; 10 ng total RNA assayed). Thus, the inductive effects of ConA and LPS on woodchuck PBMC could be differentiated using the RT-PCR assay, consistent with their expected actions on different cell types. The effects of LPS on rodent PBMC RNA induction may differ from those

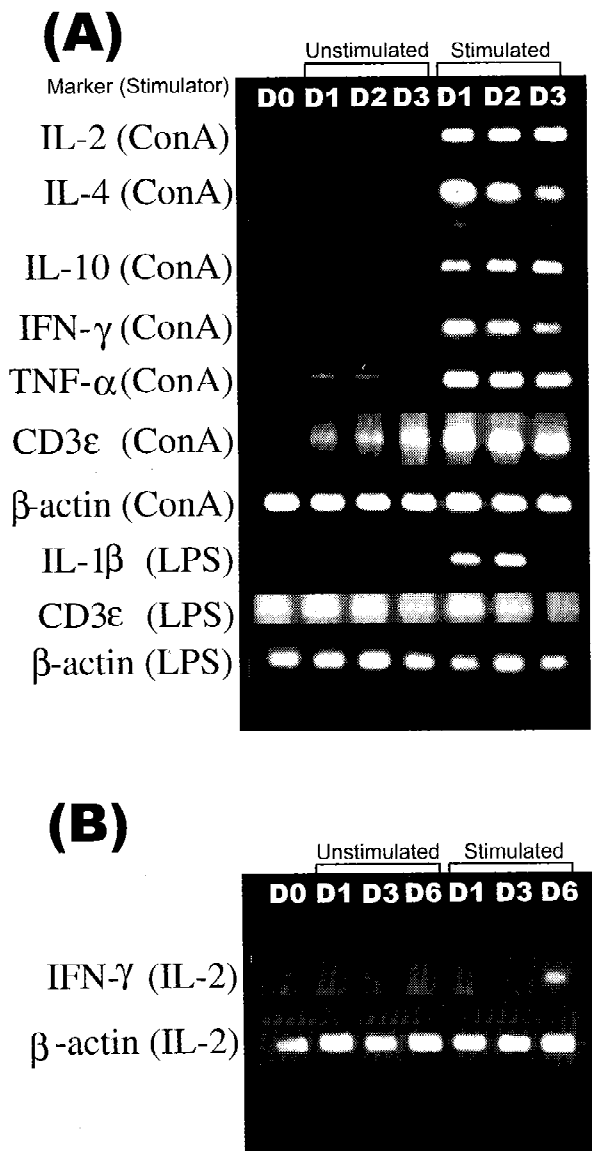

Fig. 5. Example of RT-PCR detection of woodchuck cytokine and CD3ε in cultured PBMC. Woodchuck PBMC were isolated from EDTA-treated whole blood by Ficoll-hypaque method. Total RNA was extracted at time zero and then at three time points following culture for unstimulated PBMC and for paired PBMC samples that were stimulated with ConA or LPS (Day 0 (D0), D1, D2, D3), or with human rIL2 (D0, D1, D3, D6). The amount of PBMC target RNA was normalized to the same concentration by OD_{260} for a given set of primers (see below), and also to yield approximately equivalent amounts of β-actin PCR products. The PCR products were analyzed on the agarose-gel and stained by ETBR. Only the portions of the gels containing the appropriate sized PCR product are shown. **A:** Induction by ConA and LPS; ConA [IL-2, IL-4, IFN-γ; 50 ng PBMC RNA] [IL-10, CD3, β-actin; 10 ng PBMC RNA] [TNF-α; 1 ng PBMC RNA]; LPS [IL-1β, CD3, β-actin; 10 ng PBMC RNA]. **B:** Induction of IFN-γ at day 6 by human rIL-2; rIL-2 [IFN-γ, β-actin; 10 ng PBMC RNA].

seen with human PBMC because of its added ability to stimulate proliferation of rodent B cells. However, LPS induces IFN-γ and TNF-α RNAs in all species, including the woodchuck most likely via activation of monocytes in PBMC preparations (Table I).

In another experiment, human rIL-2 induced IFN-γ RNA in woodchuck PBMC by day 6 of culture (Fig. 5B; 10 ng input RNA) (note: the original primer set was used in this experiment). Differential induction of CD3 RNA by rIL-2 was detected on day 1, but was less evident thereafter (not shown). Human rIL-2 was not as efficient as ConA for inducing CD3 (1 ng of target RNA used here). Moreover, human rIL-2 did not induce detectable woodchuck IL-2 RNA as might be expected for homologous woodchuck IL-2 (Table I). The above observations may relate to differences in the fine specificity of cross-species stimulation by IL-2.

TABLE I. Specific Induction of Woodchuck Cytokines and CD3ε in Cultured PBMC by ConA, LPS, and Human rIL-2*

	Stimulator		
Marker	ConA	LPS	Hu-rIL2
IL-1β	−	+	−
IL-2	+	−	−
IL-4	+	−	−
IL-10	+	+	−
IFN-γ	+	+	+
TNF-α	+	+	−
CD3ε	+	−	+

*RNA assayed by RT-PCR (1–100 ng depending on marker and stimulator); The plus symbol indicates specific induction of transcript in stimulated vs. unstimulated cells using criteria defined in the text associated with Figure 5. The minus sign indicates no specific induction. The woodchuck-specific internal primer pair was used to detect IFN-γ RNA in the experiments involving ConA and LPS. The original primers for IFN-γ were used to detect IFN-γ RNA in the experiments involving Hu-rIL-2.

RT-PCR Detection of Cytokine and CD3 RNA in Woodchuck Liver

Next, CD3 RNA and several immunocytokine RNAs were demonstrated in woodchuck liver tissue. For this experiment, approximately 3 μg of total liver RNA were assayed by RT-PCR, and were analyzed the products by agarose gel and staining with ETBR. The RNA specific to immunologic cells in 3 μg of liver RNA probably amounts to 1% or less of the total RNA. This will also depend on the cell type, state of activation, and degree of inflammation. However, such amounts of RNA are near the range used above to detect transcripts in stimulated and unstimulated PBMC.

Results for the RT-PCR assay of CD3 and immunocytokine RNAs in representative liver samples of uninfected and WHV-infected woodchucks are shown in Figure 6, along with β-actin controls. CD3 RNA was readily detected as a 536 bp fragment in both uninfected and infected woodchuck liver. RNA from the infected animal yielded a brighter signal under the present assay conditions. IL-1β RNA (332 bp product) was not detected in representative liver samples. IL-2 RNA was also undetected when assayed using the woodchuck-specific primers (197 bp). Detection of IL-1β and IL-2 RNAs may therefore require more total liver RNA substrate, additional PCR cycles, or conditions indicative of more extensive monocyte and/or T-cell activation in this compartment.

IL-4 (205 bp) and IL-10 (303 bp) RNAs were strongly detected in samples from uninfected and infected ani-

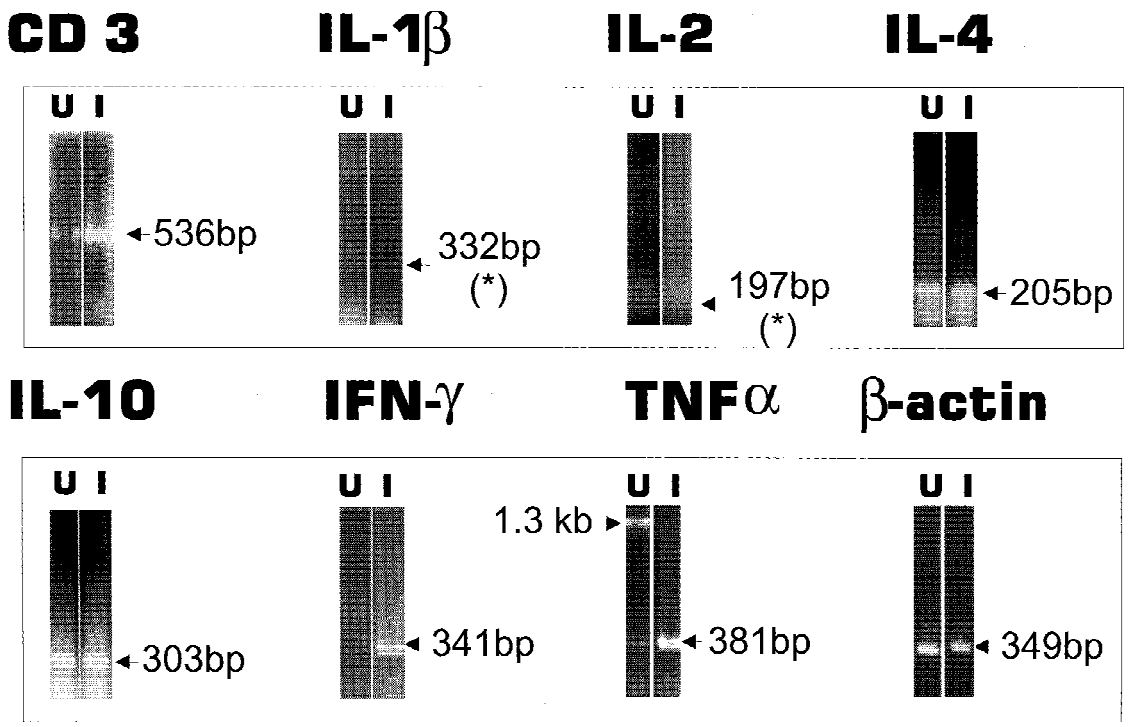

Fig. 6. Example of RT-PCR detection of cytokine and CD3ε RNA in liver from uninfected and WHV-infected woodchucks. Three micrograms of total RNA, prepared from uninfected and WHV-infected woodchuck liver, was used for RT-PCR assay of immunocytokines and CD3. RT-PCR products were analyzed on the agarose gel by staining with ETBR. Products of CD3 (536 bp), IL-4 (205 bp) and IL-10 (303 bp) were detected in both uninfected and infected animals. The product of IFN-γ (341 bp) was detected only in infected animals. The product derived from spliced TNF-α RNA (381 bp) was detected only in infected animals. The 1.3 kb product of TNF-α, whose size corresponds to that of the product from unspliced RNA, was detected in uninfected animals. U, uninfected; I, WHV-infected; *, the expected position for IL-1β (332 bp) and IL-2 (249 bp) products that were undetectable in these liver samples under present RT-PCR assay conditions.

mals (Fig. 6). Differential patterns of IFN-γ RNA expression were detected between uninfected and WHV infected woodchucks. TNF-α RNA expression also differed between uninfected and infected woodchucks; this appeared to involve processing of a higher molecular weight precursor RNA (ca. 1.3 kb) to the spliced message (381 bp) (Nakamura et al., unpublished data).

Semi-Quantitative RT-PCR Assays for Woodchuck Cytokines

In the following experiments we: 1) developed a second generation RT-PCR assay format for woodchuck cytokines involving hybridization with homologous ^{32}P-oligomers; 2) validated the absolute sensitivity of this assay using IL-2 RNA transcribed in vitro from the IL-2 clone; and 3) titrated the endpoint detection by RT-PCR of IL-2 and IFN-γ RNA units in a standardized RNA from activated PBMC. In preliminary studies by Southern blot, the respective ^{32}P-oligomers were validated as monospecific for the homologous PCR product of the expected size (data not shown).

Results for in vitro transcribed RNA from the IL-2 clone are shown in Figure 7A. The assay yielded significant positive signals for reactions containing an initial input of 100 copies or more of the target RNA; note here that only one-tenth of the reaction mixture was blotted onto the nylon filter. It may be possible to improve endpoint detection by blotting more of the final product. Under the present conditions, there was a linear relation between input RNA and hybridized counts that extended over a 4 $\log_{10}$ range.

The endpoint dilutions were determined for IL-2 and IFN-γ RNAs in a standardized RNA preparation from stimulated PBMC that contained 20 ng total RNA/μl (Fig. 7B). The $\log_{10}$ plots of hybridized counts vs. pg input of total PBMC RNA permitted quantifications over a 3 to 3.5 $\log_{10}$ range. Extrapolation of each plot to zero detection was defined as 1 unit (1 U) of target RNA. The respective endpoints for these RNAs corresponded to 1 U IL-2 RNA/3 pg total PBMC RNA and 1 U IFN-γ RNA/1 pg total PBMC RNA; the amounts in the undiluted preparation were therefore 6,600 U IL-2 RNA/20 ng total PBMC RNA and 20,000 U IFN-γ RNA/20 ng total PBMC RNA. Thus, assay of these RNA species by this method enables sensitive detection and semi-quantification of the respective RNAs using far less input RNA compared to the ETBR staining in agarose gels.

DISCUSSION

Host mechanisms that determine whether acute HBV infections resolve or proceed to chronicity involve the immune response. By analysis of the woodchuck immune response to WHV, we hope to identify or deduce molecular events that initiate resolution and chronicity, and thereby differentiate causative mecha-

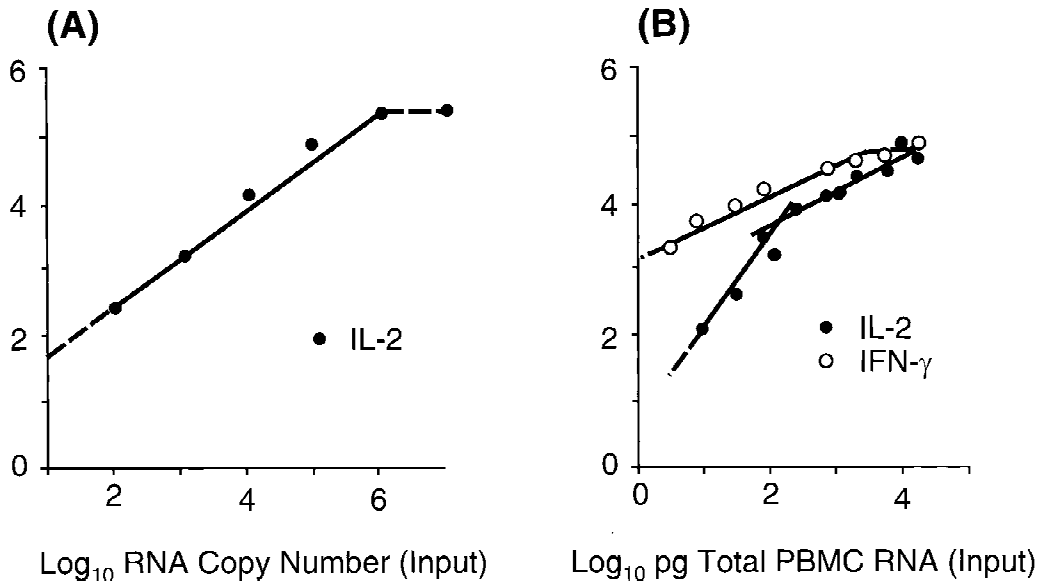

Fig. 7. Semi-quantitative RT-PCR for woodchuck immunocytokine RNAs. **A:** RNA derived from in vitro transcription of the woodchuck IL-2 plasmid was quantified by OD_{260} and serially diluted 10-fold for use as a template in RT-PCR. One-tenth (5 μl) of the PCR products were blotted onto nylon filters and hybridized to 5′-^{32}P end-labeled oligomer consisting of woodchuck IL-2 sequences homologous with the cloned cDNA. The amount of radioactivity was determined by AMBIS imaging system. **B:** Serial threefold dilutions of a standardized PBMC total RNA preparation were assayed by RT-PCR and hybridization as above using the respective primers and oligomeric probes. The primers used correspond to the respective woodchuck specific sequences underlined in Figure 1. The respective sequences used as ^{32}P-oligomeric probes are double underlined in Figure 1.

nisms from secondary effects resulting from chronic carriage. This can facilitate the development of rational immunotherapeutic approaches for eradicating established chronic HBV infection and its disease sequelae.

Continued progress in both cellular and nucleic acid-based immunologic methods are essential to further advance the woodchuck as model for immune response and inflammation in hepadnavirus infection. Basic cellular immunologic assays are available for applications in the woodchuck [Cote and Gerin, 1995; Menne et al., 1997] , but others are also needed (e.g., CTL assay). In this study, the range of assay formats was extended by developing nucleic acid-based immunologic assays for the RNAs of selected woodchuck cytokines and a T cell marker (e.g., CD3ϵ). Further development of this methodology will enable the corresponding proteins to be expressed for use in new assays, antibody development, and basic mechanistic and therapeutic studies. Moreover, cellular immunologic assays for woodchucks can also be further improved by the availability of these additional reagents and assay methods.

We isolated woodchuck-specific partial cDNA clones for the CD3 T cell marker and selected cytokines. The specificity of each clone was validated by a combination of: 1) nucleotide sequence homologies, 2) open reading frame for protein, 3) predicted amino acid sequence homologies, and 4) specific inducibility of the respective RNAs by stimulation of PBMC with mitogens (ConA, LPS) and a human cytokine (rIL-2) [Cote and Gerin, 1995]. First and second generation (semi-quantitative) RT-PCR assays were developed based on these clones that have application for detecting and estimating the relative amounts of cytokine and CD3 mRNA in woodchuck PBMC and liver. The CD3 marker is relevant for tracking of T cells. The panel of cytokine markers can potentially enable the assessment of T cell functional profiles in WHV infection, including TH1 (IL-2, IFN-γ) and TH2 (IL-4, IL-10) subsets. Other markers were considered relevant to the initiation of immune responses (IL-1β, IL-2). Still others are potentially associated with effector functions leading to the inhibition of hepadnavirus replication (e.g., IFN-γ, TNF-α). cDNAs for the heterodimeric proteins of woodchuck IL-12 were cloned previously and sequenced by the group at Roche, which should enable study of this important initiatory cytokine in WHV infection and therapy.

Continued analysis of cytokine interactions and basic immune mechanisms in HBV infection [Peters, 1996] and other models [Ahmed and Gray, 1996; Chisari, 1995; Chisari and Ferrari, 1995; Fearon and Locksley, 1996; Forsthuber et al., 1996; Gilles et al., 1992; Guidotti et al., 1994a, 1994b; Guilhot et al., 1993; Ridge et al., 1996; Sarzotti et al., 1996] represent promising approaches to determine the causes of hepadnaviral chronicity. RT-PCR-based assays can be used to study immunocytokine RNA expression in PBMC and hepatic compartments where they may be expressed in potentially very limiting amounts. This also applies when only limited tissue sampling is possible; for example, circulating lymphocytes from very young woodchucks (1–4 wk of age) or serial needle biopsies of liver.

Methods to measure directly woodchuck cytokines are not developed to the same extent as in humans and mouse models. RT-PCR of immunocytokine RNAs is a proven method for detecting small amounts of RNA

and RNA-related events at relevant sites of infection and immune response [Sieling et al., 1994; Svetic et al., 1991, 1993; Yamamura et al., 1992; Farges et al., 1995; Kim et al., 1995; Larrea et al., 1996], including in human liver tissues with hepatitis of viral and non-viral etiology [Shindo et al., 1996; Fukuda et al.,1995] and in mouse liver transplant models [Farges et al., 1995]. We developed the present RT-PCR assays for woodchuck immunocytokines and CD3 using total RNA from stimulated woodchuck PBMC in which a high proportion of the cells were activated. Differential RNA expression was evident when these preparations were compared with those from unstimulated PBMC. In many cases, the first generation assays could even detect and differentiate the low levels of cytokine RNAs in unstimulated woodchuck PBMC. The first generation RT-PCR assays were also able to detect many of these markers in liver as well, which is relevant to future applications.

The liver is the major site of infection and inflammation in WHV (and HBV) infection. It was therefore necessary to show detection of lymphocyte-specific RNAs in that compartment, such as CD3, which is T cell specific. Other key cytokines, with the exceptions of IL-1β and IL-2, were also detected readily, and prospects for enhancing detection levels by second generation assays appear promising. The proportion of activated lymphocytes or macrophages in a given compartment affects differential detection. The differential detection of RNA using PBMC and liver from uninfected and WHV-infected woodchucks depends on the differential level of RNA expression and processing. During hepatitis, most of the inflammatory cells and Kupffer cells in liver will be activated, which should facilitate differential detection of some immunocytokine RNAs in uninfected and infected woodchucks, as indicated for IFN-γ and TNF-α (Fig. 6). This will enable retrospective and prospective analyses for such differences in resolved and chronic WHV infections, and the identification of cell types in which these processes occur.

In conclusion, we developed woodchuck-specific partial cDNA clones for selected immunocytokines and the CD3 marker of T cells. Nucleic acid primers were used in RT-PCR assays to detect the RNAs for these markers in PBMC and liver. Continued study of the woodchuck immune response to WHV infection using these assays will provide insight into the immune mechanisms that initiate and maintain chronic hepadnavirus infection and disease and, hence, enable development of improved immunotherapies for established chronic HBV infection.

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